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The Effect of Intracellular Ca^{2+} on GABA-activated Currents in Cerebellar Granule Cells in Culture

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Abstract. The patch clamp technique was used to study the effects of intracellular free calcium ($[Ca^{2+}]_i$) on $GABA_A$-evoked whole-cell and single channel currents of cultured cerebellar granule cells. Changes in $[Ca^{2+}]_i$ were obtained by adding to the extracellular solution the calcium ionophore A23187 (2 μM). The relationship between $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$ in the presence or absence of A23187 was assessed using fluorimetric measurements from Fura-2 loaded cells. In 2 mM $[Ca^{2+}]_o$ and A23187, $[Ca^{2+}]_i$ was about 1.5 μM, whereas in the absence of A23187 it was about 250 nM. In whole-cell experiments (symmetrical chloride concentrations) at −50 mV, GABA (0.5 μM) evoked inward currents that did not desensitize. Bath application of A23187 significantly reduced the steady-state amplitude of GABA currents by 37 ± 6%. Single channel currents activated by GABA (0.5 μM) were also recorded in the outside-out configuration of the patch clamp technique. Kinetic analysis of single channel events revealed that A23187 significantly increased the long closed time constant (τ_{c3}) without affecting the open time constants (τ_{o1} and τ_{o2}) or the short and medium closed time constants (τ_{c1} and τ_{c2}). Moreover, application of A23187 induced a significant reduction of burst duration (τ_b). We conclude that a rise in $[Ca^{2+}]_i$ by A23187 may decrease the binding affinity of GABA for the $GABA_A$ receptor.

Key words: Patch clamp — $GABA_A$ receptor — Fura-2 — Intracellular Ca^{2+} — Rat cerebellum — A23187

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Introduction

γ-Aminobutyric acid (GABA) is the main inhibitory transmitter in the mammalian central nervous system. This amino acid inhibits neuronal firing by activating two pharmacologically distinct receptor types, named $GABA_A$ and $GABA_B$, coupled respectively to Cl^- and cationic channels (Sivilotti & Nistri, 1991). The $GABA_A$ receptor is a multisubunit ligand-gated ion channel (for a review, *see* Wisden & Seeburg, 1992). The subunit composition of this channel determines its functional properties. The gating of $GABA_A$-receptor channels can be modulated by several agents acting either from the extracellular or the intracellular side of the membrane.

Several reports have demonstrated a crucial role of intracellular Ca^{2+}, $[Ca^{2+}]_i$, in the regulation of $GABA_A$-receptor function (Stelzer, 1992). An increase of cytosolic calcium inhibits GABA-evoked currents in intermediate lobe cells (Taleb et al., 1987). The same effect was observed in acutely dissociated CA1 hippocampal pyramidal neurons (Stelzer et al., 1988; Chen et al., 1990). Chen and co-workers (1990) found that an elevation of $[Ca^{2+}]_i$ accelerates the rundown of GABA responses. These authors suggested that $[Ca^{2+}]_i$ probably shifts the balance of $GABA_A$, maintaining the phosphorylation/dephosphorylation cycle toward dephosphorylation by activating the calcium-dependent phosphatase, calcineurin. In bullfrog sensory neurons, the short-term elevation of $[Ca^{2+}]_i$ produced by the activation of voltage-gated Ca^{2+} channels suppressed GABA-activated currents (Inoue et al., 1986). This effect was explained as a decrease in apparent affinity of the $GABA_A$-receptor in the presence of increased $[Ca^{2+}]_i$. Furthermore, in a preliminary study Beherends et al. (1988), using the inside-out configuration of the patch clamp technique on frog sensory neurons, showed that different calcium con-

centrations applied to the inner side of the membrane reduced the single channel open times and increased the closed times.

The mechanism of modulation of $GABA_A$-receptor channel gating by $[Ca^{2+}]_i$ is still unclear. A more detailed kinetic study focused on burst analysis will help to understand the fine mechanisms of this modulation.

In this study, we investigated the effects of changes in $[Ca^{2+}]_i$ on $GABA_A$-activated whole-cell and single channel currents using both microfluorimetric and electrophysiological (patch clamp) techniques. It was found that a rise in $[Ca^{2+}]_i$ depressed nondesensitizing GABA-evoked currents and induced a significant reduction of burst duration without affecting the fast channel gating.

Materials and Methods

Cell Preparation

Neurons were isolated from cerebella of one-week-old Wistar rats using a standard procedure (Levi et al., 1984). Minced cerebellar tissue was treated with trypsin and then mechanically dissociated. At the final stage, cerebellar granule neurons were plated with basal Eagle medium (Irvine) containing L-glutamine and fetal calf serum (10%). The cells were plated on petri dishes or, for fluorimetry experiments, on flasks (Falcon) and kept in the incubator at 37°C and 5% CO_2. Under these conditions the cells were maintained for about 10 days and were available for experiments starting from the first day in culture (DIC).

Microfluorometry

Cultured granule cells were detached from flasks by trypsin (0.05%)-EDTA (0.02%) treatment for 5 min. After centrifugation, the cells were resuspended and then incubated in DMEM with 5 μM Fura-2-acetoxy methylester (Molecular Probes, Eugene, OR) for 1 hr at 37°C. The cells were then rinsed with buffer (in mM; 140 NaCl, 5 KCl, 1.5 $MgCl_2$, 5 glucose, 10 HEPES, 0.1% bovine serum albumin fraction V (Sigma); pH 7.4 with TRIS) and resuspended at a concentration of 5×10^5 cells/ml. To obtain different Ca^{2+} concentrations, a few microliters of a stock $CaCl_2$ solution were added. Fluorescence was measured under continuous stirring in a Jasco spectrofluorimeter. Signal calibration was performed according to Meldolesi et al. (1984).

Electrophysiological Experiments

The patch clamp technique was used to study the effect of $[Ca^{2+}]_i$ on GABA-activated whole-cell and single channel currents (Hamill et al., 1981). Borosilicate glass pipettes were coated with Sylgard (Corning) and fire-polished to get a tip resistance of 5–10 MΩ (in working solutions). The external (bath) solution contained (in mM): NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES-NaOH 10, D-glucose 20. The pipette was filled with a solution containing (in mM): CsCl 138, $MgCl_2$ 4, ATP-Na_2 2, HEPES-KOH 10. The pH of both solutions was adjusted to 7.4. ATP and Mg^{2+} were included in the pipette solution to prevent an eventual rundown of GABA-activated currents (Stelzer et al., 1988). Cells were continuously superfused with control solution through a one-barrel pipette. GABA (0.5 μM) was applied by pressure (~1 psi) through a separate borosilicate glass pipette (internal diameter ~5 μm), placed at about 50 μm from the patched cell. GABA was delivered by pneumatic pressure system (PPS-2, Medical System). The same device was used to control the duration and the pressure of the GABA ejection. With this technique, the response to GABA appeared within 50 msec after the activation of the pressure system. This time was much shorter than the desensitization time constant of the response to 1 μM GABA (Kilić, Moran & Churbini, 1993). To increase the concentration of intracellular Ca^{2+}, the same procedure was used as in Fura-2 experiments. The calcium ionophore A23187 (2 μM) was directly added to the bath solution via the superfusing system.

GABA-evoked currents were studied using a standard patch clamp amplifier (EPC-7, List Medical Instruments). The current signal filtered at 10 kHz was recorded on a videotape recorder. A microcomputer (Atari 1040ST) and D/A converter (ITC-16, Instrutech) were used to control the potential in the pipette. Stored data were filtered with a Butterworth filter (Krohn-Hite 3202) at a cut-off frequency of 1–2.5 kHz and then transferred to the microcomputer by a 16-bit A/D converter (ITC-16, Instrutech) at a sampling time of 200–500 μsec for single channel events and 10 msec for whole-cell currents. Experiments were performed at room temperature (22–24°C). GABA and A23187 were purchased from Sigma.

Data Analysis

The time and amplitude analysis of single channel currents were performed with the TAC program (Instrutech) which uses 50% threshold criteria in the detection of channel opening. Open and closed time histograms were fitted with exponential functions by the method of Sigworth and Sine (1987). The probability of opening *Np* in excised patches was calculated as the ratio between the sum of all open times and the total observation time, taking the duration of GABA application as the total observation time.

The groups of channel openings that are separated by gaps, all shorter than some specified length, are defined as bursts of openings. The critical time t_c that defines a burst was determined by the following equation (Colquhoun & Sakmann, 1985):

$$1 - \exp(-t_c/\tau_l) = \exp(-t_c/\tau_m) \quad (1)$$

where τ_l and τ_m are the long and intermediate time constants in the closed time distributions. The mean burst length was obtained from the fitting of burst duration histograms by single exponentials.

Statistical analysis was made using the Sigma-Plot program (Jandel). Wilcoxon test was used for comparison of paired data. Results are expressed as mean ± SEM.

Results

$[Ca^{2+}]_i$ Measurements

A microfluorimetric technique was used to measure $[Ca^{2+}]_i$ in the presence of different concentrations of extracellular calcium $[Ca^{2+}]_o$. Granule cells loaded with Fura-2 (5 μM) were exposed to increasing concentrations of external Ca^{2+} (from 10 μM to 2 mM, $n = 4$). $[Ca^{2+}]_i$ concentration was only weakly dependent upon external $[Ca^{2+}]_o$. As shown in Fig. 1*B*, $[Ca^{2+}]_i$ varied from 202 ± 19 nM in the presence of 10 μM $[Ca^{2+}]_o$ to 253 ± 18 nM in 2 mM $[Ca^{2+}]_o$. To increase $[Ca^{2+}]_i$, we added the calcium ionophore A23187 to the external medium. In the

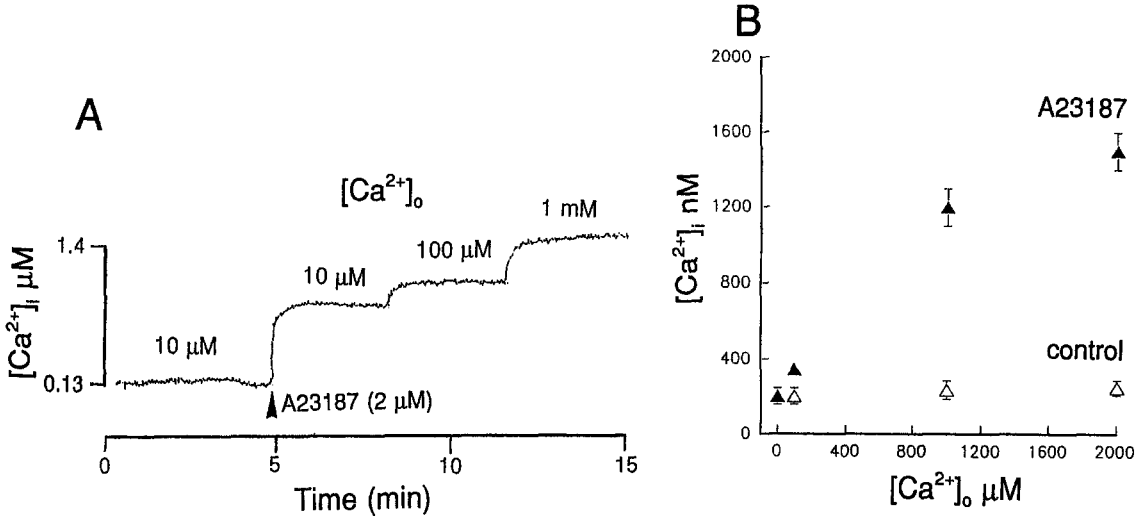

Fig. 1. Effects of A23187 on $[Ca^{2+}]_i$ as a function of $[Ca^{2+}]_o$ in Fura-2-loaded cerebellar granule cells. (*A*) Changes in intracellular calcium as a function of extracellular calcium concentrations (indicated above the traces). A23187 (2 μM) was added at the arrow. Notice that in A23187, after addition of Ca^{2+} to the external medium, $[Ca^{2+}]_i$ reached a steady-state level in approximately 30 sec. (*B*) Steady-state values of $[Ca^{2+}]_i$ as a function of $[Ca^{2+}]_o$ measured in the absence (△) or presence (▲) of A23187 ($n = 4$). In this and the following figure, bars represent SEM.

presence of A23187 (2 μM) in the bathing solution containing 0.01 mM of calcium, $[Ca^{2+}]_i$ increased slowly and reached a steady-state level approximately 30 sec after the beginning of perfusion with A23187. The rise in $[Ca^{2+}]_i$ by A23187 was found to be dependent on $[Ca^{2+}]_o$ in a nonlinear way. In the example of Fig. 1*A*, $[Ca^{2+}]_i$ was 218 nM in 0.01 mM $[Ca^{2+}]_o$, whereas it was 347 and 1,380 nM in 0.1 and 1 mM $[Ca^{2+}]_o$, respectively. Steady-state values of $[Ca^{2+}]_i$ measured in the presence or absence of A23187 were statistically different ($P < 0.01$).

Activation of different receptor types by neurotransmitters may produce an increase in $[Ca^{2+}]_i$ (Connor, Tseng & Hockberger, 1987, Mulle et al., 1992). Therefore, we tested the levels of $[Ca^{2+}]_i$ upon application of low concentrations of GABA. GABA (0.5–1 μM) did not change $[Ca^{2+}]_i$.

The same experimental approach used to rise intracellular calcium by A23187 was used in electrophysiological experiments to study the modulation of GABA currents by $[Ca^{2+}]_i$.

Electrophysiological Experiments

GABA-evoked Whole-Cell Currents

The currents activated by GABA in cerebellar granule cells in culture have been already fully characterized (Cull-Candy & Ogden, 1985; Cull-Candy & Usowicz, 1989; Kilić et al., 1993). As already shown, total GABA currents desensitize in a concentration-dependent way (Akaike, Inoue & Krishtal, 1986; Oh & Dichter, 1992). In preliminary experiments we found that a low concentration of GABA (0.5 μM) activated, at a holding potential of −50 mV, a nondesensitizing whole-cell current which could be repeatedly reproduced for prolonged periods of time (up to 20 min, Fig. 2*B*). The current reached a steady-state value of 200–400 msec after the beginning of GABA application and remained constant throughout the time of GABA application (10–30 sec).

In the presence of A23187 (2 μM) GABA currents were significantly ($P < 0.01$) reduced (37 ± 6%, $n = 9$) but could still be repeatedly reproduced without any rundown for prolonged periods of time (Fig. 2). It should be noticed, however, that A23187 did not affect the leak conductance, the time to peak or the decay time of GABA response. The decrease in amplitude of GABA currents in the presence of A23187 may be due to two independent processes: (i) rundown mediated via Ca^{2+}-dependent proteins; (ii) direct interaction between Ca^{2+} ions and $GABA_A$-receptor channel complex. To further analyze this problem, GABA-activated single channel events were studied in the same experimental conditions. To get stationary responses, only a low, nondesensitizing concentration of GABA was used.

GABA-evoked Single Channel Currents

The outside-out configuration of the patch clamp technique was used to study single channel currents activated by GABA. In agreement with a previous report (Kilić et al., 1993), in the presence of 2 mM $[Ca^{2+}]_o$, GABA (0.5 μM) activated single channel events having a main conductance of 31 pS (Fig. 3); occasionally a subconductance level of 19 pS was found. The current/voltage relation was linear in the potential range from −60 to 50

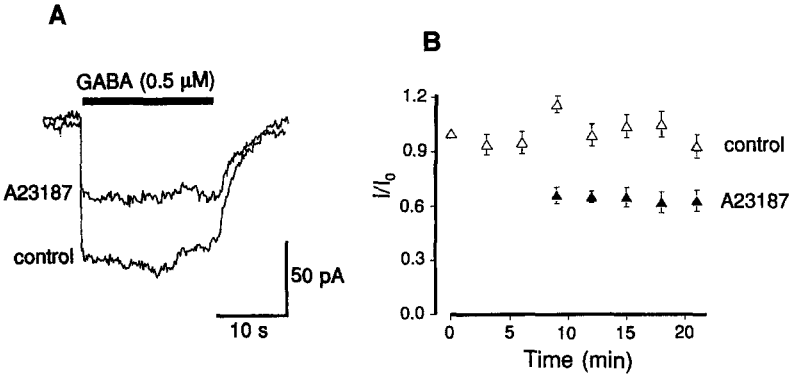

Fig. 2. Reduction of the amplitude of GABA-evoked whole-cell current by A23187. (*A*) Two superimposed traces of whole-cell currents evoked by GABA (0.5 μM, bar) in the absence or presence of A23187 (2 μM) at a holding potential of −50 mV. No change in the offset was observed after addition of the ionophore. (*B*) Plot of the amplitude of normalized whole-cell currents elicited every 3 min by application of GABA (0.5 μM) *vs.* time, in control condition (n = 9, △) and during superfusion with A23187 (2 μM, n = 9, ▲). GABA currents were normalized to the first response obtained at time 0 in the absence of A23187. A23187 was applied 7 min after the first response.

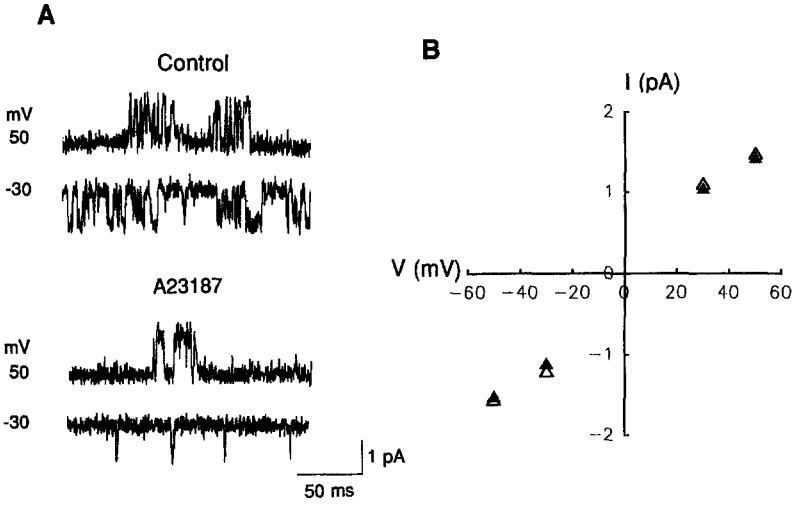

Fig. 3. A23187 reduces the open probability of GABA-evoked single channel currents without changing the single channel conductance. (*A*) Single channel currents activated by GABA (0.5 μM) in an outside-out patch, recorded at two different holding potentials (−30 and +50 mV) in control conditions and in the presence of A23187 (2 μM). Bandwidth: 1 kHz. (*B*) Single channel current/voltage relationship of the main conductance state for the records shown in *A*, in the absence (△) or presence (▲) of A23187 (2 μM).

mV and, as expected for symmetrical chloride solutions, the reversal potential was about 0 mV (Fig. 3*B*). In the presence of A23187 (2 μM), the single channel conductance did not change. The linear voltage dependence of GABA currents persisted and the reversal potential did not change (Fig. 3*B*). However, a rise in $[Ca^{2+}]_i$ by A23187, induced a significant ($P < 0.06$) decrease of the probability of single channel opening by 50 ± 6% (Fig. 4; Table). This finding is consistent with the data obtained in whole-cell configuration in the same experimental conditions. Furthermore, since the property of stationarity was preserved at the single channel level in control or in the presence of A23187 (*not shown*), the current amplitude of the steady-state of whole-cell currents may be directly proportional to the probability of opening of single channel currents. A detailed analysis of the kinetic properties provided further information on the effects of $[Ca^{2+}]_i$ on GABA-activated single channel currents. Open time histograms were well fitted with two time constants, whereas closed time histograms were well fitted with three time constants (Fig. 5; Table). The calcium ionophore A23187 did not significantly change mean open times, fast and intermediate closed time constants (τ_{c1} and τ_{c2}), but it significantly ($P < 0.06$) increased the long closed time constant τ_{c3} (Fig. 5, Table). Therefore, the fast transitions from closed to open states were not affected by the elevated $[Ca^{2+}]_i$. Moreover, the ratio of the various components to the total area was unchanged in both experimental conditions (Table). On the other hand, the probability of opening (P_o) was dependent on the long closed time constant (τ_{c3}). Figure 6 shows the correlation between the probability of opening (Np) and the three different time constants in control conditions (*A*) and in the presence of A23187 (*B*).

At this point an attempt was made to analyze GABA-induced burst activity to get more information about the modulation of the gating properties of the $GABA_A$-receptor channel by $[Ca^{2+}]_i$. For this purpose, the critical time that delineates the bursts was calculated using Eq. (1) taking τ_l and τ_m as τ_{c3} and τ_{c2}, respectively. Although, as shown in the Table, only an eightfold difference exists between τ_{c2} and τ_{c3}, we analyzed only those cases (n = 7) in which the ratio between τ_{c2} and τ_{c3} was more than tenfold. Figure 6*C* shows the relation between the critical time t_c and the probability of opening Np in control conditions or in the presence of

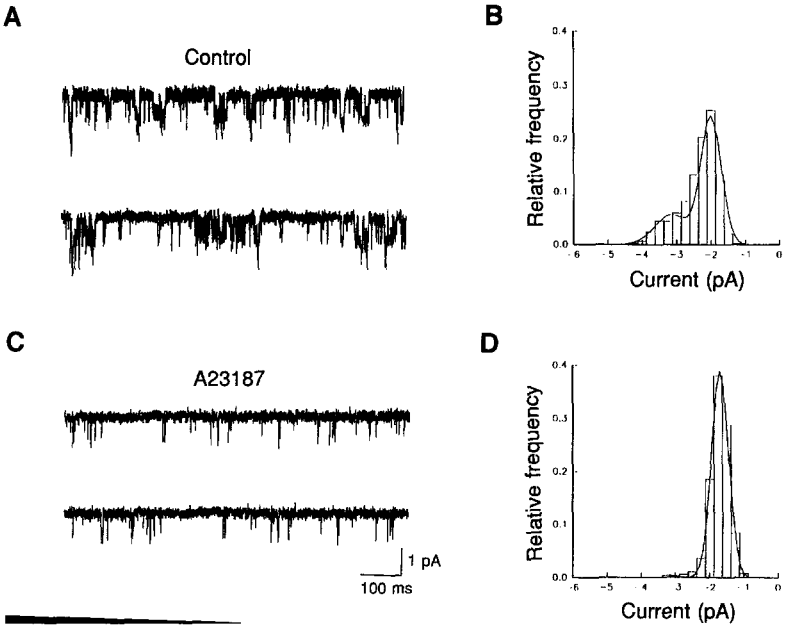

Fig. 4. A23187 reduces the open probability of GABA-activated single channel currents in an outside-out membrane patch. Single channel currents activated by GABA (0.5 μM) at a holding potential of −50 mV, in control conditions (*A*) and in the presence of A23187 (2 μM, *C*). Bandwidth: 1 kHz. Amplitude histogram of GABA-activated single channel events at a holding potential of −50 mV, for the records shown in *A*, in the absence (*B*) or presence (*D*) of A23187. This cell (like four others) did not fulfil the criteria used to define burst length in the two experimental conditions (τ_l / $\tau_m \geq 10$ and similar *Np*) and therefore was not included in the total number of cells analyzed for burst duration.

Table 1. Open (τ_o) and shunt (τ_c) time constants of single channel currents activated by 500 nM GABA ($V_h = -50$ mV) in control conditions and in the presence of A23187

	Control	A23187 (2 μM)
τ_{o1}	0.91 ± 0.11 (11)	0.67 ± 0.18 (11)
Area (%)	79.6 ± 3.8	84.1 ± 3.6
τ_{o2}	4.20 ± 0.85 (11)	3.10 ± 1.35 (11)
Area (%)	20.4 ± 3.7	15.9 ± 3.9
τ_{c1}	0.75 ± 0.24 (11)	1.30 ± 0.50 (11)
Area (%)	33.0 ± 3.9	31.0 ± 4.4
τ_{c2}	4.70 ± 0.82 (11)	6.40 ± 1.25 (11)
Area (%)	38.3 ± 3.9	40.7 ± 5.3
τ_{c3}	38.1 ± 11.07 (11)	72.2 ± 13.4 (11)*
Area (%)	28.8 ± 4.7	27.3 ± 5.3
τ_b	20.7 ± 6.4 (7)	11.6 ± 2.5 (7)**
Np	0.16 ± 0.03 (11)	0.08 ± 0.02 (11)*

The areas under the fitted curves are normalized to 1. Below each time constant value the corresponding area contributing to the histogram is shown. The number of experiments is indicated in parentheses. *$P <$ 0.06; **$0.05 < P < 0.1$.

A23187. As shown in the figure, longer critical times were associated with the lower probability of opening. The inverse relation between t_c and *Np* persisted also for the corresponding burst duration τ_b (Fig. 6*D*). Obviously such dependence of τ_b on *Np* can lead to wrong conclusions since the probability of opening was significantly changed in the presence of A23187. To make a comparison of burst length in the two experimental conditions, only the cases with $\tau_l/\tau_m \geq 10$ and similar *Np* were considered. In these cases we found a significant ($0.05 < P < 0.1$) decrease in burst length (Table) during superfusion with A23187. This difference was dependent on the probability of opening being more pronounced for the low values of *Np*.

Discussion

The main finding of the present study is that a rise of intracellular calcium by the calcium ionophore A23187 reduced the amplitude of the nondesensitizing whole-cell GABA current and the probability of opening of single channel without changing the fast channel gating.

GABA-evoked Whole-Cell Currents

The present findings are in agreement with previous reports in which changes in intracellular calcium concentration induced a depression of the peak amplitude of GABA currents. In these studies the rise of $[Ca^{2+}]_i$ was triggered by calcium entry either through voltage-dependent calcium channels (Inoue et al., 1986), *N*-methyl-D-aspartate or nicotinic receptors (Stelzer et al., 1987; Mulle et al., 1992). A depression of GABA currents following a sustained increase in cytosolic calcium by caffeine was also reported (Desaulles, Boux & Feltz, 1991; Mouginot, Feltz & Schlichter, 1991). Intracellular calcium exerts several effects on GABA currents. It may either depress or potentiate them depending on its concentration. Taleb et al. (1987) found that the maximal activity of GABA currents was achieved at a cytoplasmic free calcium concentration ranging from 10^{-8} to 10^{-7} M. At both extremes the activity was reduced. This is in

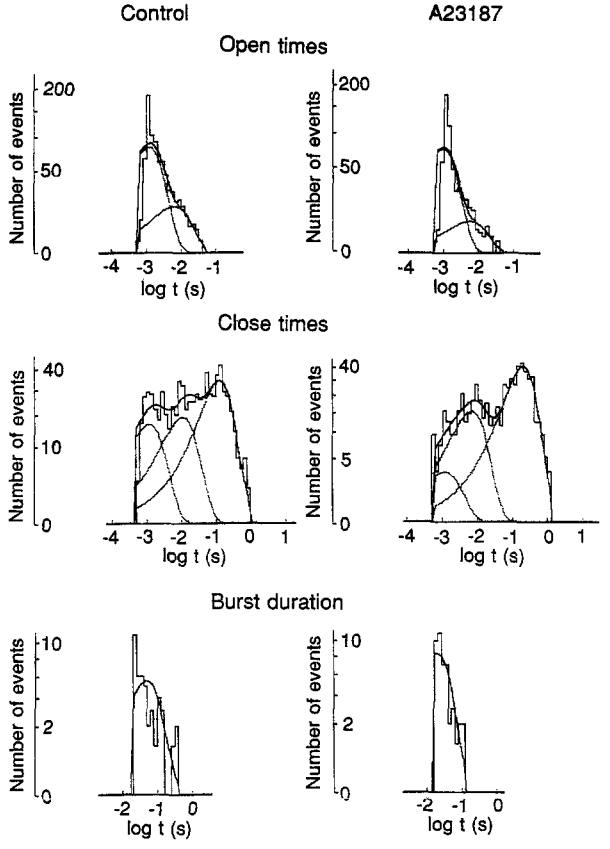

Fig. 5. The calcium ionophore A23187 reduces the long closed time and burst duration of GABA-evoked single channel currents. Open time, closed time and burst duration histograms of single channel events activated by GABA (0.5 μM) at a holding potential of −50 mV in an outside-out patch from a cerebellar granule cell in control conditions (left) or in the presence of A23187 (2 μM, right). The histograms are displayed with square-root transformed ordinates. The mean shut and open times as well as burst duration histograms were fitted with exponential functions according to Sigworth and Sine (1987). The best fit values (in msec) in control conditions were: τ_{o1} 1.2; τ_{o2} 6.7; τ_{c1} 1.3; τ_{c2} 11.4; τ_{c3} 132; τ_b 50. In A23187 were: τ_{o1} 0.98; τ_{o2} 5.9; τ_{c1} 1.3; τ_{c2} 7.0; τ_{c3} 196; τ_b 20.

agreement with the results of Mouginot et al. (1991) who showed a bell-shaped relationship between the concentration of $[Ca^{2+}]_i$ and the activity of the $GABA_A$-receptor-gated Cl^- channel. In this framework, an up-regulation of GABA current by calcium entry through a train of depolarizing pulses, as that described by Llano, Leresche and Marty (1991) in cerebellar Purkinje cells, also could be explained. In our experimental conditions, loading of calcium was achieved by superfusion of the calcium ionophore A23187 which has been successfully used to modulate calcium-dependent activity (Kudo, Takeda & Yamasaki, 1990; Grandolfo et al., 1992). The actual concentration of $[Ca^{2+}]_i$ present before or after A23187 was assessed by microfluorimetric techniques. Using this method we found, in agreement with other studies (Connor et al., 1987; Parks et al., 1991), that Fura-2 loaded cerebellar granule cells maintained, in control conditions, a low level of $[Ca^{2+}]_i$ (around 100 nM). This level increased upon superfusion of A23187 to a maximum value higher than 1 μM in the presence of 2 mM $[Ca^{2+}]_o$. It should be stressed, however, that this value probably reflects an underestimation of $[Ca^{2+}]_i$ present in electrophysiological experiments, because of the lack of a calcium-buffering system in patched cells and the diffusion of calcium from the cell into the pipette. Furthermore, we found that low concentrations of GABA, as those used in our electrophysiological experiments, did not produce any measurable change in $[Ca^{2+}]_i$.

The decrease in the amplitude of whole-cell GABA current by increasing levels of intracellular calcium was not due to an enhanced GABA desensitization since the inactivation kinetics of GABA currents were the same in the presence or absence of A23187. The lack of effects of $[Ca^{2+}]_i$ on total current kinetics (*see also* Marchenko, 1991) suggests that the time course of the probability of opening of GABA-activated single channel events remained the same in control or in the presence of A23187. As already mentioned, a rundown of GABA currents occurs when $GABA_A$ receptors are deprived of their physiological intracellular environment (Stelzer, 1992). This process, which can be prevented by addition of ATP and Mg^{2+} to the intracellular medium and by buffering intracellular calcium to low levels by calcium chelating agents, depends on the competition between a phosphorylation process that maintains and a Ca^{2+}-dependent process that reduces the Cl^- conductance (Chen et al., 1990). A rundown process, triggered by a rise in $[Ca^{2+}]_i$, may contribute to the depression of Cl^- conductance in the presence of A23187. In our experimental conditions (with ATP and Mg^{2+} in the pipette), GABA currents could be repeatedly elicited unchanged for prolonged periods of time even in the presence of A23187. Since an enzymatic activation would lead to a progressive rundown, the lower level plateau reached by GABA currents in the presence of the ionophore is in favor of a direct effect of calcium ions on the $GABA_A$-receptor channel complex as suggested by Inoue and co-workers (1986). According to these authors, $[Ca^{2+}]_i$ may in fact change the affinity of GABA for its receptor, as shown by the competitive inhibition of GABA-activated whole-cell currents by I_{Ca}. However, a full comprehension of this phenomenon could be achieved only by a kinetic analysis of elementary currents activated by GABA in outside-out patches obtained in the same experimental conditions as for whole-cell experiments.

GABA-evoked Single Channel Events

From the single channel data, it seems unlikely that the action of $[Ca^{2+}]_i$ on GABA takes place at the level of the

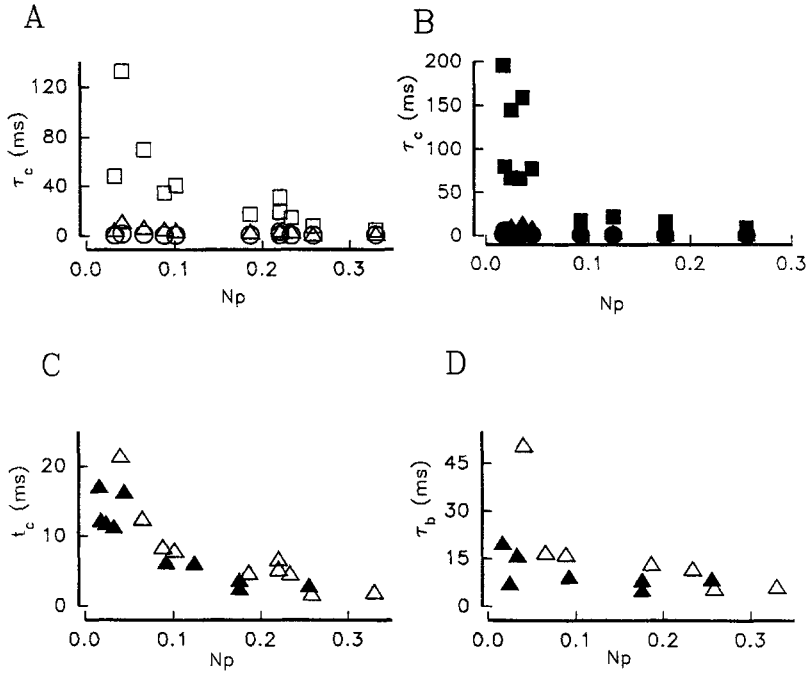

Fig. 6. (*A* and *B*) Plot of the different closed time constants (τ_c) *vs.* open probability (*Np*) of GABA-activated single channel events, in control conditions (n = 11, open symbols, *A*) and during application of A23187 (n = 11, filled symbols, *B*). Each symbol represents a different experiment (○,●) τ_{c1}; (△,▲) τ_{c2}; (□,■) τ_{c3}. Note that the longer time constant (τ_{c3}) depends on the probability of opening (*Np*). (*C* and *D*) Plot of critical time (t_c) and burst duration (τ_b) *vs.* open probability of GABA-activated single channel currents (same experiments as in *A* and *B*), in the presence (▲) or absence (△) of A23187. Notice that longer critical times and longer burst duration are associated with lower probability of openings (*Np*).

channel pore itself: in fact, the unitary conductance or the mean open time constants were found to be the same in both experimental conditions (in the presence or absence of A23187). From the kinetic analysis of τ_o and τ_c, it seems plausible that $[Ca^{2+}]_i$ affects the gating properties of $GABA_A$ channels. In the presence of A23187, the probability of openings of unitary GABA currents decreased by about the same amount as found in whole-cell currents (~50%). This decrease was probably due to an increase of the longer closed time constants τ_{c3}. An increase in closed time distribution was also found by Beherends et al. (1988) in bullfrog sensory neurons in the inside-out configuration, but in contrast to our findings, the increase in τ_c was associated with a calcium-dependent decrease in the duration of the open time of GABA-gated Cl^- channels. However, these authors did not discriminate between single openings and burst activity, resulting from clustering of several openings. In our experiments, the short closed time constants τ_{c1} and τ_{c2} were independent of $[Ca^{2+}]_i$. This, together with the invariability of the mean open times, suggests that the fast transitions of the activated $GABA_A$-receptor channel are not changed in the presence of high concentrations of intracellular calcium. Assuming the general del Castillo-Katz model (del Castillo & Katz, 1957)

$$A + R \rightleftharpoons AR \rightleftharpoons AR^*$$

where A is agonist, R is receptor, AR is the activated receptor in the closed state and AR^* is the activated receptor in open conformation, it is likely that the transitions between AR and AR^* in the model are not influenced by cytosolic Ca^{2+}. Furthermore, assuming that the mean burst duration is inversely proportional to the dissociation constant of agonist for its receptor (Colquhoun & Hawkes, 1983), the decrease in burst length observed in the presence of A23187 may be due to a decrease in the binding affinity of GABA for the receptor.

In conclusion, our results confirm the previous assumption of Inoue et al. (1986) that an increase in $[Ca^{2+}]_i$ decreases the binding affinity of GABA for the $GABA_A$-receptor channel. This, in concomitance with a calcium-dependent rundown process, may be responsible for the depression of GABA currents by an increase in cytosolic calcium observed in our experimental conditions.

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